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TITLE: Neuroprotective Strategies for the Treatment of Blast-Induced Optic Neuropathy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Traumatic optic neuropathy is a rare but devastating injury that can result from blunt force or explosive blast. Presentation can be delayed by weeks and patients can ultimately lose vision completely in the affected eye. Unfortunately, in the military, bilateral injuries are more common. We use a mouse model of closed globe trauma to induce indirect traumatic optic neuropathy in order to test underlying mechanisms with the goal of identifying therapies for this currently untreatable blinding condition. We have identified that the IL-1 pathway is causative to the secondary neurodegeneration after trauma. We have measured the release kinetics of EPO-R76E packaged microparticles for intraocular delivery. We are currently analyzing results from the galantamine treatment studies including electroretinogram, visual evoked potential, and optical coherence tomography. We detect no change in acetylcholine levels after blast in our repeat injury model. This could suggest that the cholinergic neurons are not particularly susceptible, or that there are important molecular differences between single large blast and repeat lower blast pressure injuries.

15. SUBJECT TERMS

None listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION: A major limiting factor to the development of treatments for indirect traumatic optic neuropathy has been the absence of a suitable animal model for: (1) mimicking the initial injury; and (2) tracking secondary degeneration. We have addressed this limitation in an innovative way, by developing an experimental system that models ocular blast injury. This system recapitulates many of the same injuries detected in Service Members with blast-induced ocular trauma, including retinal detachments, optic nerve atrophy, and vision loss. We will assess the efficacy of two therapeutic agents in our model of blast-induced traumatic optic neuropathy. Our model causes early oxidative stress, neuroinflammation and inner retinal dysfunction followed by decreased vision and optic nerve degeneration. This suggests that degeneration of the retinal ganglion cell (RGC) axons in the optic nerve is a secondary event. Secondary degeneration of downstream neurons is well described in the central nervous system (CNS) after trauma. One such example within the visual system is degeneration in the lateral geniculate nucleus after lesion of the optic nerve or ocular hypertension. Therefore, **our study has implications for neurodegenerations from trauma extending beyond optic neuropathy.**

2. KEYWORDS:
retinal ganglion cell (RGC), traumatic optic neuropathy, inflammasome, erythropoietin (EPO), electroretinogram (ERG), visual evoked potential (VEP), interleukin-1 (IL-1)

3. ACCOMPLISHMENTS:
What were the major goals of the project?
Aim 1: Elucidate the cellular mechanisms underlying visual dysfunction after blast. *We will test the working hypothesis that blast activates pyroptosis in starburst amacrine cells causing decreased signaling to the dsRGCs, which leads to dendritic pruning and axon degeneration in the dsRGCs.*
Major Task 1: Obtain approval for mouse studies.
Major Task 2: Assess if pyroptosis is activated after blast. Months 5-12
Major Task 3: Assess if signaling from starburst amacrine cells is altered by blast. Months 12-14
Major Task 4: Determine if the dendritic trees of the dsRGCs are altered by blast. Months 5-24

Aim 2: Assess the efficacy of galantamine in preventing neurodegeneration secondary to blast. *We will test the hypothesis that galantamine will restore signaling to the dsRGCs thus preventing their degeneration after blast.*
Major Task 1: Assess vision in treated and control blast mice. Months 17-24
Major Task 2: Assess histology of treated and control blast mice. Months 20-28
Major Task 3: Quantify neurochemical changes in the retina. Months 20-21

Aim 3: Assess if reduction in neuroinflammation and oxidative stress by EPO-R76E prevents neurodegeneration secondary to blast. *We will test the working hypothesis that EPO-R76E will protect against blast-induced optic neuropathy by limiting oxidative stress and neuroinflammation.*
Major Task 1: Assess vision in treated and control blast mice. Months 28-34
Major Task 2: Assess histology of treated and control blast mice. Months 30-36
Major Task 3: Quantify EPO-R76E levels. Months 30-32

What was accomplished under these goals?
1) Major Activities:

A. We have demonstrated that the pyroptotic pathway, otherwise known as the inflammasome or IL-1 pathway, plays a key role in the secondary degeneration after ocular blast injury. The pathway is activated after blast exposure and can be inhibited by treatment with antioxidants. Our data shows the mitochondrially-derived superoxide plays a key role in blast-induced activation of the IL-1 pathway. Further, the antioxidants prevent axon degeneration and vision loss. We are currently writing the results and plan to submit the manuscript before the end of the year.

B. We measured retinal acetylcholine (ACh) levels and detected no change between sham and blast injured eyes at 1 or 4 months after injury. Further, treatment with galantamine did not change the levels of ACh. These analyses were performed on repeat blast rather than single blast mice. Therefore, this data could suggest either that there is a difference in cellular response mechanism depending on the injury type/magnitude, or that cholinergic neurons and cholinergic signaling are not sensitive to ocular trauma.

C. We are in the midst of analyzing optic nerve histology and superior colliculus fluorescence (mice were intravitreally injected with fluorescently labeled CTB) from galantamine treated and control mice to determine if this treatment preserved axons and axon transport, respectively. VEPs are also being analyzed.

D. We detected no change in systemic cytokines (blood samples) in mice on a ketogenic diet, suggesting that this diet had no effect on the systemic inflammatory state of the body and any effect we see with the diet is due to action in the eye.

E. An increase in retinal superoxide levels due to blast injury was prevented by the ketogenic diet.

F. We have generated and performed *in vitro* testing of inherently antioxidant microparticles that are loaded with EPO-R76E. We will begin testing them in vivo in the next quarter.

2) Specific Objectives:

A. To complete our study on the role of the inflammasome pathway in optic nerve degeneration after blast-induced ocular trauma.

B. To finish the Drd4.eGFP mouse breeding, resulting in a line of mice that can be used for the proposed studies.

C. To initiate studies on the efficacy of galantamine in protecting against degeneration and vision loss after blast-induced ocular trauma.

D. To develop clinically translatable intraocular delivery of EPO-R76E.

3) Significant Results:

Blocking ROS and the IL-1 pathway prevents axon degeneration and vision loss after ocular trauma.

As previously reported, we detect an increase in peroxynitrite after blast, as indicated by increased immunolabeling for 3-nitrotyrosine (**Fig. 1**). Peroxynitrite is produced by a reaction between superoxide and nitric oxide. These reactive oxygen species (ROS) can feed into the pyroptotic (a.k.a. inflammasome or IL-1) pathway as seen in Figure 1. Last year we reported that after blast there was an increase in cleaved caspase-1, IL-1α, IL-1β, and IL-18, indicating that blast exposure activates this pathway in the retina. This year we extended these findings to determine if there was a relationship between the oxidative stress and the IL-1 pathway, and if either was causative to the secondary axon degeneration and vision loss that occurs after ocular blast trauma.

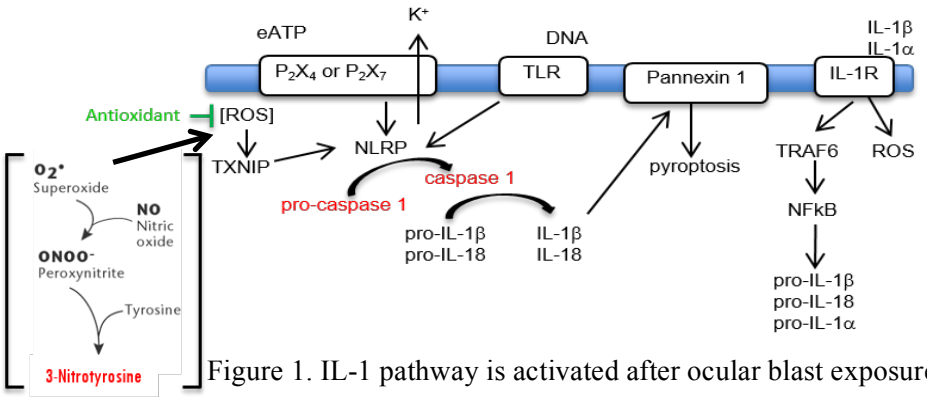
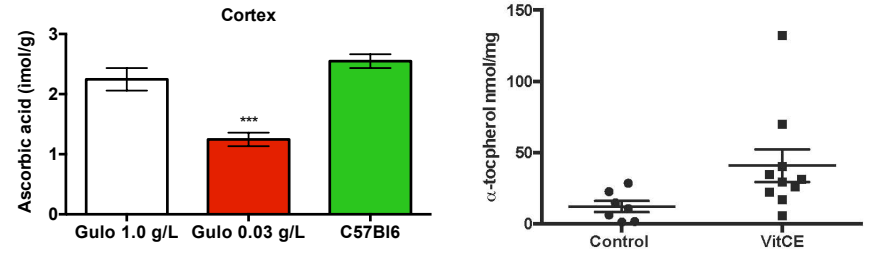


Figure 1. IL-1 pathway is activated after ocular blast exposure.



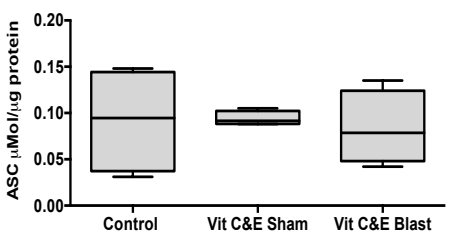


Figure 2. Tissue levels of VitC and VitE under different dietary conditions. A) VitC in Gulo$^{-/-}$ mice on high or low VitC water. B) VitE in retinas from mice on control or high VitCE diet. C) Vit C levels in retinas from mice on control or high VitCE diet.

We either increased or decreased the antioxidant capacity of the retina to assess the effect of ROS on the IL-1 pathway and the optic

neuropathy and vision loss. Mice generate their own Vitamin C through the enzyme L-gulolactone oxidase (Gulo). To decrease the antioxidant capacity of the mice, we obtained Gulo knock-out (Gulo$^{-/-}$) mice and maintained them on low Vitamin C (VitC) water. To increase the antioxidant capacity, we fed wild-type mice a high Vitamin C and high Vitamin E diet (VitCE). These diets altered tissue levels of VitC and VitE as predicted (**Fig. 2**). VitC (ascorbate) levels were decreased in Gulo-/- mice maintained on lowVitC water (0.03g/L) and VitE (α-tocopherol), but not VitC levels were increased in C57Bl/6 mice fed a high VitCE diet. The VitC levels are not increased because it is utilized intracellularly to recycle the VitE. We measured superoxide levels in vivo in the retina by injecting DHE into the vitreous and quantifying the fluorescence elicited by DHE upon reaction with superoxide (**Fig. 3A**). Injury caused an increase in superoxide at 2 and 4 weeks after blast in control and low VitC mice. Mice maintained on the high VitCE diet did not have elevated superoxide. A major source of superoxide is the mitochondria, which produces superoxide as a by-produce of oxidative phosphorylatio n. This superoxide is normally detoxified by superoxide dismutase 2 (SOD2). We detected a decrease in total

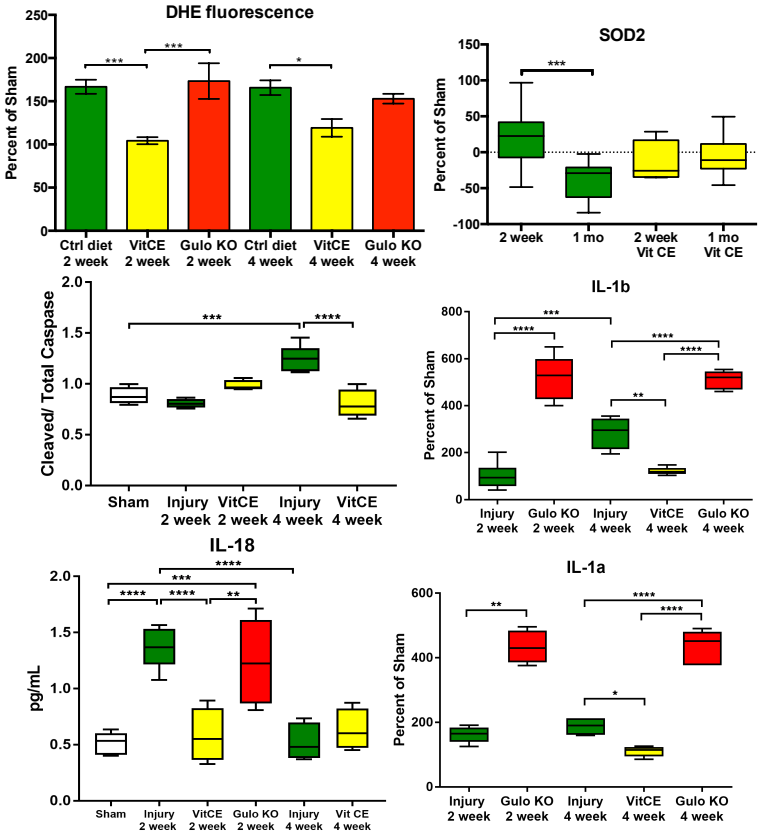


Figure X. A) Antioxidants blocked increase in superoxide levels (DHE fluorescence) and B) decrease in SOD2 levels after blast. C-F) Antioxidants prevent activation of the IL-1 pathway.

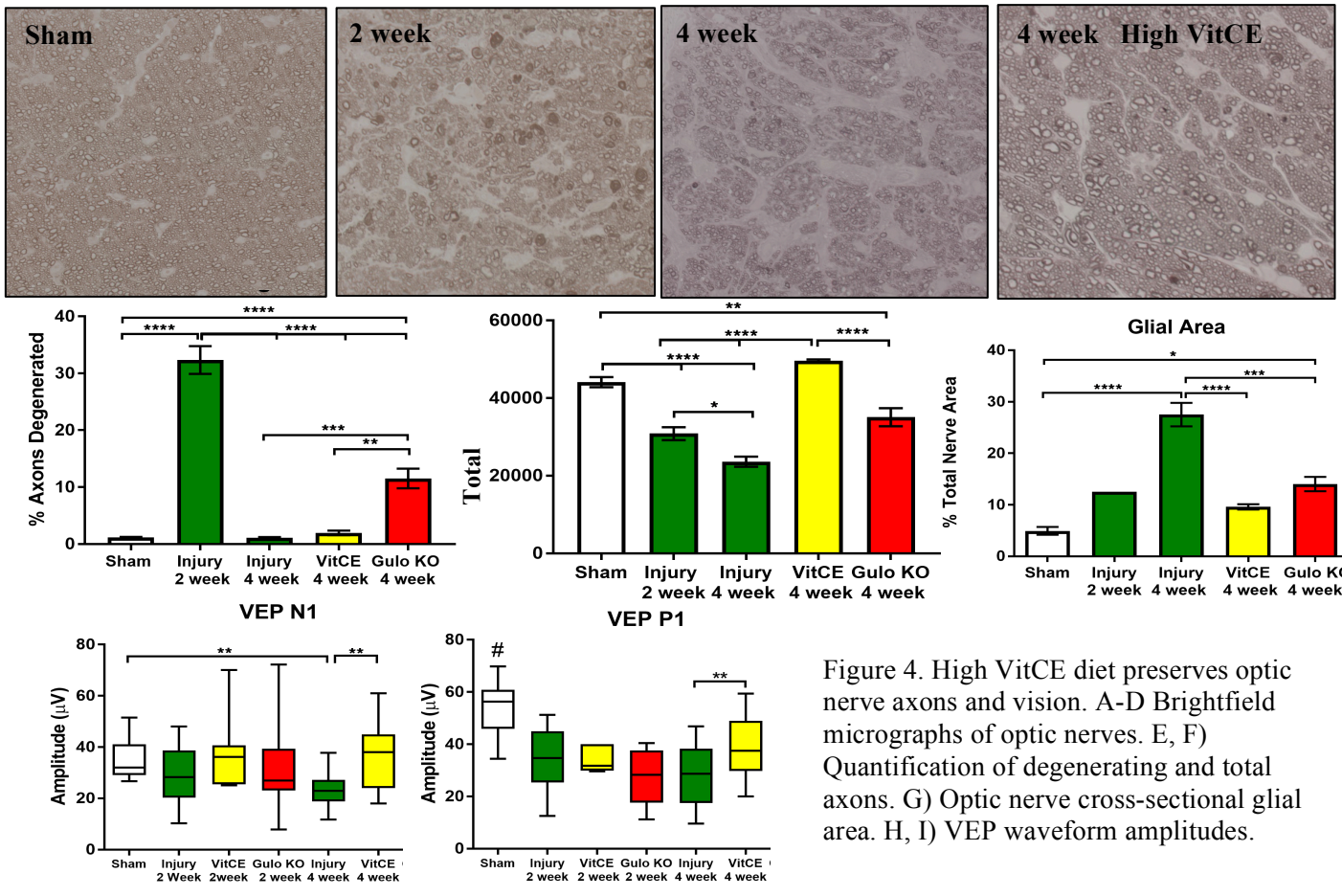


Figure 4. High VitCE diet preserves optic nerve axons and vision. A-D Brightfield micrographs of optic nerves. E, F) Quantification of degenerating and total axons. G) Optic nerve cross-sectional glial area. H, I) VEP waveform amplitudes.

SOD2 levels at 4 weeks after blast (**Fig. 3B**). This decrease was prevented by the high VitCE diet.

We next assessed the effect on the IL-1 pathway. The levels of cleaved caspase-1 were not increased in retinas from mice on the high VitCE diet (Fig. 4A). Levels of IL-1a and IL-1b were increased in mice on a low VitC diet (Fig. 4B,C). Finally, the levels of IL-1a, IL-1b, and IL-18 were similar to sham controls in the post-blast mice treated with the high VitCE diet (Fig. 4B-D). **Therefore, blocking the increase in ROS also blocked activation of the IL-1 pathway.**

Finally, we assessed the effect of decreasing ROS and blocking the IL-1 pathway on optic nerve degeneration and vision. We are still in the midst of analyzing the two-week data and are adding mice to the Gulo$^{-/-}$ group. Despite that, our data shows protection of the high VitCE diet at 4 weeks after blast by both optic nerve histology (Fig. 4X) and quantification of the visual evoked potential (VEP) (Fig. 4X). Sham mice had an average of 44,137 axons in the optic nerve. At 4-weeks after blast this was reduced to only 23,609 axons, a 46% decrease from sham. In contrast, mice treated with the high VitCE diet had an average of 49,656 axons in their optic nerves. In addition, our data shows that the majority of the axon degeneration occurs at two weeks after trauma in our repeated blast paradigm; 30% of the total 46% axon loss occurred at this time point (**Fig. 4E**).

Galantamine

We previously reported a decrease in GABA levels at 3 months after blast. We also previously published an increase in caspase-1 immunolabeling in the cholinergic amacrine cells, which co-release GABA, after blast (ref). This provided the rationale for testing the efficacy of galantamine, a nicotinic AChR agonist, which also causes an increase in GABA signaling in other models. We had not directly measured ACh levels in the retina after blast. Here we show that at 1 and 4 months after blast there is no change in ACh levels (Fig. 5). Further, treatment with galantamine had no effect on total ACh levels suggesting that the retina was already working at maximal capacity. This could be due to the shift from a single 26psi blast to a repeated 15psi blast paradigm, or it could suggest that GABA, but not ACh is altered after blast. The HPLC protocols for measuring ACh and GABA are not compatible. We will measure GABA in the next cohort of mice.

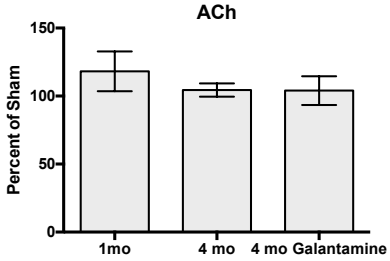


Figure 5. ACh levels in the retina at 1 and 4 months after blast.

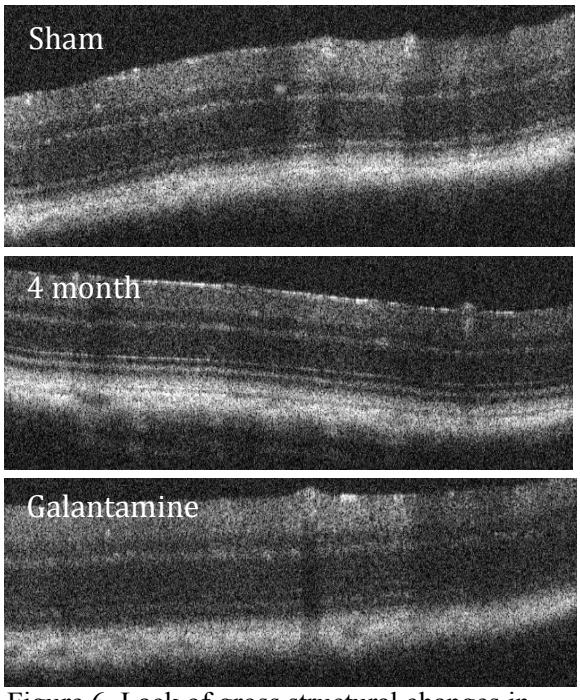


Figure 6. Lack of gross structural changes in the retina 4 months after blast.

We performed optical coherence tomography at baseline and post-blast. The OCT scans do not appear to detected a difference between sham, blast, and blast plus galantamine groups at the 4 month time-point (Fig. 6). However, we have not performed a regional analysis nor made quantifications of the layers as of yet. The images do suggest that the photoreceptors are spared, which matches the ERG data in this repeat blast model. The collected tissue is being processed for histological analysis. We expect to have the analysis completed next quarter. We are currently analyzing baseline, 2 month, and 4 month post-blast ERG and VEP data on galantamine and control mice.

We have generated a colony of Drd4.eGFP mice. We have crossed them onto the C57Bl/6 background and selected for mice that: 1) retain eGFP, 2) do not carry the retinal degeneration mutation found in the original Drd4.eGFP line, 3) retain a genetic marker of the C57Bl/6 line, and 4) have a black coat. We have three litters of mice and are currently checking their genotype to confirm successful generation of useful mice. Depending on the results of the genotyping we expect to begin using these mice for the dendritic arborization studies either the next quarter or early next year.

Erythropoietin

We have generated more inherently antioxidant microparticles and have loaded them with EPO-R76E and demonstrated release *in vitro*. We plan to begin testing the efficacy of this delivery system *in vivo* in the next quarter with results obtained and analyzed next year.

Ketogenic Diet

We measured *in vivo* levels of superoxide in the retina by injecting DHE intravitreally at one month after blast. We detect an elevation in DHE fluorescence as compared to sham (**Fig. XA**). This elevation is prevented by treatment with a ketogenic diet (**Fig. XA**). In contrast we have not detected a decrease in retinal levels of IL-1α or IL-1β with this diet (data not shown). We are currently processing the optic nerves and superior colliculus to assess axon integrity and axon transport, respectively.

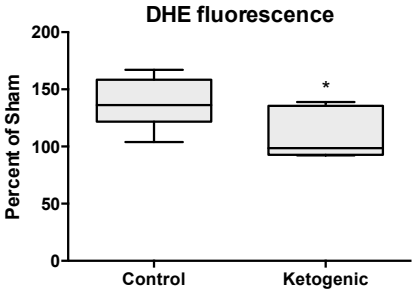


Figure X. Ketogenic diet prevents blast-induced increase in superoxide.

4) Other Achievements:

Finally, as part of our Translation Plan, a pre-application was submitted to the Department of Defense Vision Research Program and was invited for a full application. The premise of the proposal is to test the efficacy of IL-1 pathway inhibitors given after blast in preventing neuronal degeneration and vision loss. The grant will be submitted October 25th. I am also a collaborator on a clinical trial DoD VRP grant application to test the safety of a Fas inhibitor (which would block the IL-1 pathway).

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

We recently presented portions of these findings at the annual meeting for the Association for Research in Vision and Ophthalmology. We are now writing up a manuscript that will be submitted to a peer-reviewed journal.

What do you plan to do during the next reporting period to accomplish the goals?

1. We will test the efficacy of the EPO-R76E containing microparticles *in vivo* in our model.
2. We will finish analyzing the data from the ketogenic diet and galantamine treated mouse studies. If necessary we will repeat groups. We expect to need one more cohort for the galantamine study.
3. We will expose Drd4.eGFP mice to blast and perform confocal microscopy on the flat-mounted retinas.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have identified that the inflammasome/IL-1 pathway is causative to the secondary axon degeneration and vision loss after ocular trauma. There are several druggable targets in this pathway that could be therapeutically efficacious for the treatment of indirect traumatic optic neuropathy. We are submitting a grant application to the DoD VRP to test the efficacy of inhibitors of the priming or activation of this pathway.

What was the impact on other disciplines?

The retina is part of the CNS and therefore what we learn in these studies is also applicable to brain and spinal cord injury.

What was the impact on technology transfer?

In the current study we have packaged a form of EPO with attenuated erythropoietic activity (EPO-R76E) into nanoparticles to provide sustained but reversible treatment *in vivo*. Successful completion of this project may well yield a clinically translatable product. Both the nanoparticles and EPO-R76E are novel. Our

collaborator, Dr. Duvall, has connections to pharmaceutical companies whom he has licensed other nanoparticles to in the past, so we have a path to the market and to the clinic.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:
Changes in approach and reasons for change:
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them:
Nothing to Report.

Changes that had a significant impact on expenditures:
Nothing to Report.

Significant changes in use or care of vertebrate animals:
Nothing to Report.

6. PRODUCTS:
Publications, conference papers, and presentations:
Publications:
None
Conference Papers:
1. Bernardo-Colon A, Clark A, Rex TS. (2017) Ocular trauma induces sterile inflammation. 6th Military Vision Symposium on Ocular and Vision Injury; federal support acknowledged.

2. Watkins D, Bernardo-Colon A, Rex TS. (2017) A blast device for inducing ocular trauma in mouse models. 6th Military Vision Symposium on Ocular and Vision Injury; federal support acknowledged.

3. Clark A, Bernardo-Colon A, Rex TS. (2017) Ocular trauma induces sterile inflammation. 57: ARVO E-Abstract 1762; federal support acknowledged.

4. Rex TS. (2017) Inhibiting sterile inflammation protects against indirect traumatic optic neuropathy, Annual Meeting Association for Research in Vision and Ophthalmology (ARVO) Ocular Trauma Symposium, Baltimore, MD; federal support acknowledged

5. Vest V, Bernardo-Colon A, Li Z, Clark A, Clifton J, Rex TS. (2017) Repeat lower magnitude trauma induces greater axon degeneration: treatment with antioxidants. J Neurotrauma Suppl. 10241; federal support acknowledged

Presentations:
1. Rex TS. (2017) Mechanisms and therapy for traumatic optic neuropathy, SUNY, Brooklyn, NY; federal support acknowledged

Website or other internet site:
Nothing to Report

Technologies or techniques:
Nothing to Report

Inventions, patent applications, and/or licenses:
Nothing to Report.

7. PARTICIPATNS & OTHER COLLABORATING ORGANIZATIONS:
What individuals have worked on the project?

Name: Tonia S. Rex
Project Role: PI
Researcher Identifier (ORCID ID): 0000-0002-2566-8723
Nearest person month worked: 5
Contribution to Project: Supervised all activities, designed studies, trained lab members, published and presented research.
Funding Support:

Name: Alexandra Bernardo
Project Role: RA III
Researcher Identifier (ORCID ID): 0000-0001-7384-6187
Nearest person month worked: 12
Contribution to Project: Lead researcher for all experiments. Designed and performed experiments, trained Zhu Li.
Funding Support:

Name: Zhu Li
Project Role: RA III
Researcher Identifier (ORCID ID): N/A
Nearest person month worked: 8
Contribution to Project: Performed some animal experiments and biochemical analyses.
Funding Support:

Name: Marcus Colyer
Project Role: Consultant
Researcher Identifier (ORCID ID): N/A
Nearest person month worked: N/A
Contribution to Project: Dr. Colyer was key in helping me form the relationships with Ophthalmologists and the Intrepid Center at Fort Campell that were key to the Clinical Study on TBI that I was recently awarded through the DoD. Due to Dr. Colyers' invitation, I presented again this year on blast physics and biomechanics during the Tri-Service Ocular Trauma Course for military Ophthalmology residents at the Uniformed Services Health Sciences University. I am helping to organize a new laboratory session on ocular blast trauma in this course and will be traveling back up there the first week of November to meet with the leader for the new session.
Funding Support:

Name: Craig Duvall
Project Role: Collaborator
Researcher Identifier (ORCID ID): 0000-0003-3979-0620
Nearest person month worked: N/A
Contribution to Project: Taught the RAIII how to develop microparticles and package EPO-R76E into them. Helped with the *in vitro* release kinetics measurements.
Funding Support:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
W81XWH-17-2-0055 DoD, CDMRP
Role: PI (Tonia Rex); 20% effort Total Costs: $2.0M 2017-2020
Title: Quantitative Evaluation of Visual and Auditory Dysfunction and Multi-Sensory Integration in Complex TBI Patients
The goals of the project are to: 1) identify a sensitive quantitative structural diagnostic for visual and auditory dysfunction after TBI, 2) identify a sensitive quantitative functional diagnostic for visual and auditory dysfunction after TBI, and 3) quantify audio-visual integration in complex TBI patients.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS:
None.

9. APPENDICES:
See attached updated Quad Chart.

Neuroprotective strategies for the treatment of blast-induced optic neuropathy

MR141315

W81XWH-15-1-0559

PI: Tonia S. Rex **Org:** Vanderbilt University Medical Center **Award Amount:** $1.5 million

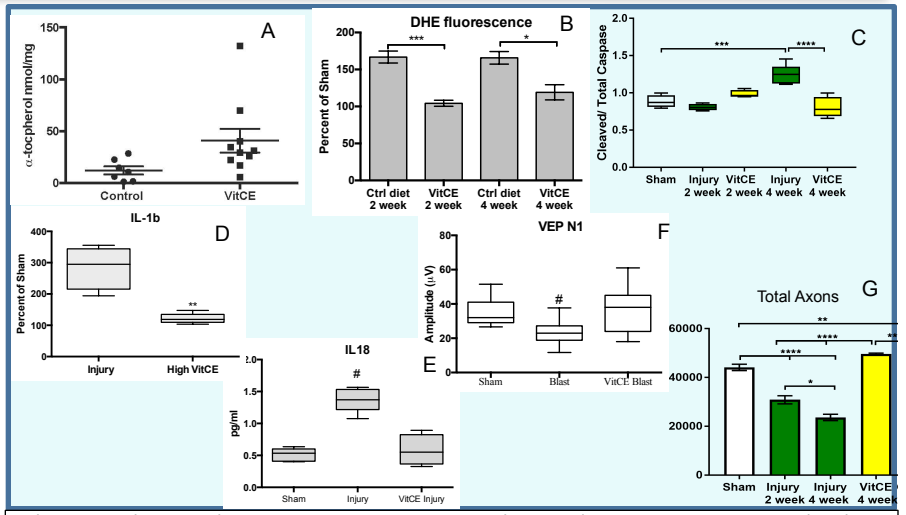
Study/Product Aim(s)

- We hypothesize that blast-induced optic nerve degeneration and vision loss is due to oxidative stress and neuroinflammation, which causes cholinergic neuron dysfunction.
- Aim 1: We will test the working hypothesis that blast activates inflammation-mediated cell death in the cholinergic amacrine cells and leads to decreased signaling to the direction-selective retinal ganglion cells and degeneration of their axons.
- Aim 2: We will test the working hypothesis that restoration of signaling to the retinal ganglion cells by treatment with galantamine will preserve the optic nerve and vision after blast.

Aim 3: We will test the working hypothesis that a non-erythropoietic form of erythropoietin (EPO-R76E) will block oxidative stress and neuroinflammation and preserve the optic nerve and vision after blast.

Approach

We will use our model of blast induced optic neuropathy to assess the efficacy of galantamine and erythropoietin. We will quantify relevant neurotransmitters, oxidative stress, neuroinflammation, axon transport, histology, and vision.



The IL-1 pathway and ROS are causative to TON. High VitCE diet increases tissue VitE levels (A), decreases ROS (B), blocks the IL-1 pathway (C-E), and preserves vision (F) and optic nerve axons(G) after repeat blast.

Timeline and Cost

Activities CY	15	16	17	
Specific Aim 1				
Specific Aim 2				
Specific Aim 3				
Estimated/Actual Budget ($K)	**$305**	**$515**	**$680**	

Updated: 10/11/17

Goals/Milestones

CY16 Goal – Determine the role of inflammation-mediated cell death on blast induced vision loss and axon degeneration.

☑Quantify levels of ACh after blast

❑ Measure the dendritic fields of the retinal ganglion cells

CY17 Goal – Determine the efficacy of galantamine

☐ ☑ Quantify vision and optic nerve histology – in process.

☐ ☑ Quantify secondary outcome measures. Need to repeat.

CY18 Goal – Determine the efficacy of EPO-R76E microparticles on blast induced optic neuropathy.

☐ Quantify vision, and optic nerve histology in treated and control mice.

☐ Quantify secondary outcome measures in treated and control mice.

☐**Comments/Challenges/Issues/Concerns**

- Timelines and spending are behind due to closing of the animal facilities last year.

Budget Expenditure to Date

Projected Annual Expenditure: $506,000

Actual Annual Expenditure: $514,592